International Journal of Medicine and Pharmaceutical Sciences (IJMPS)
ISSN(P): 2250-0049; ISSN(E): 2321-0095
Vol. 4, Issue 4, Aug 2014, 67-76

IN VITRO INHIBITORY EFFICACY OF CRANBERRY FRUIT ON *ESCHERICHIA COLI* AND *PROTEUS MIRABILIS* ISOLATED FROM UTI PATIENTS IN HILLA CITY

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ABSTRACT

Background: Use of prolong antibiotics in UTI cases may cause severe side effects and resistance in bacteria against antibiotics. Therefore, we need to alternative herbal and non-antibiotic therapy for UTI such as cranberry fruit.

Purpose: Aim of the study is to better understand effects of dried cranberry powder on certain local uropathogenic strains of *Escherichia coli* and *Proteus mirabilis* in cases of urinary tract infection.

Methods: The cranberry powder (CP) was obtained from W.N pharmaceuticals, Canada. The bacterial isolates were isolated and identified according to diagnostic features as golden characterization of morphology and biophysiology for *Escherichia coli* and *Proteus mirabilis*. The antibacterial activity of CP was done against motility, adherence and biofilm formation, also compared with some UTI antibiotics.

Results: Present study suggests the cranberry have antibacterial properties against *E.coli* and *Pr.mirabilis*. The MIC values for *E.coli* and *Pr.mirabilis* were 1.2500 and 0.1563 mg /ml respectively. The ability of both genera to adherence to epithelial cells was decreased when exposed to CP. The mean adherence was decrease from 20 to 1.9 bacteria per cell. The efficacy of biofilm formation by *E.coli* and *Pr.mirabilis* was much reduced after 24 hours. The motility of these bacteria was completely inhibited following CP exposure to 10 mg/ml, and temporally inhibited by 5 mg/ml after 18 hr.

Conclusions: The powder of cranberry fruit has potential antibacterial activity against uropathogens; *E.coli* and *Pr. mirabilis*, and it can be using in preventing UTIs.

KEYWORDS: Cranberry Powder, *Vaccinium macrocarpon*, UTI, *E. coli* and *Proteus mirabilis*

INTRODUCTION

Urinary tract infections (UTIs) are an important health concern with approximately 150 million cases of UTIs occurring each year on a global basis with correspondingly significant morbidity and associated healthcare costs estimated at $1.6 billion[1]. The urinary tract is one of the most common sites of bacterial infections in humans. UTI is defined by a certain threshold number of bacteria in the urine (normally 10^4 to 10^5 CFU per ml)[2].

Eighty percent of urinary tract infections (UTIs) in humans are caused by Gram-negative, rod-shaped, flagellated and facultative anaerobic bacteria of the family Enterobacteriaceae with name *Escherichia coli* and *Proteus* species[3,4].

In patients with recurrent urinary tract infections (UTIs), long-term antimicrobial prophylaxis is indicated. This method is effective but can cause adverse reactions and can increase emergence of antimicrobial resistance. Therefore, the need for alternative therapies for UTI prophylaxis is evident. Cranberries are one non-antibiotic alternative therapy [5].

Cranberries are the fruit of a native plant of North America. These red berries are used in foods and in herbal products. Historically, cranberry fruits and leaves of *Vaccinium macrocarpon* were used for a variety of health problems, such as wounds, urinary disorders, diarrhea, diabetes, stomach ailments, and liver problems [6]. In other article [7], cranberry has been used as a folk or traditional remedy for urinary tract infections or *Helicobacter pylori* infections that can lead to stomach ulcers, or to prevent dental plaque. Cranberry has also been reported to have antioxidant and anticancer activity [8]. The cranberries are used to produce beverages and many other food products, as well as dietary supplements in the form of extracts, capsules, or tablets [8]. One study compared cranberries with twenty other fruits, showing that cranberries had a high amount of total polyphenols antioxidants. Cranberry tannins have laboratory evidence for anti-clotting properties and may prevent recurring urinary tract infections in women; however, a review of available research concluded that there is little evidence to support the efficacy of cranberry products in treating UTIs. Long-term tolerance is also an issue [9].

The main goal of the work reported here was to study the effects of a cranberry powder (CP) on *Escherichia coli* and *Proteus mirabilis* isolated from UTI patients and to assess it effect on bacterial motility, adherence to uroepithelial cells, biofilm formation and other virulence that have important role in pathogenesis of these bacteria in UTI.

MATERIAL AND METHODS

Preparation of Cranberry Solution

The dried fruit powder of American cranberry, *Vaccinium macrocarpon*, was obtained from WN pharmaceuticals company, Canada. Cranberry powder (CP) solution was prepared according manufacturer directions in different concentrations, and allowed to stand for 72 hr, and sterilized by filtration using Millipore (0.45 micrometer) filter paper. The cranberry powder solution stored in sterile bottles and kept at 4^{o}C until further use for screening of antimicrobial activity.

Bacterial Isolates

Escherichia coli and *Proteus mirabilis* were isolated from clinical cases of UTI patients at teaching Hilla hospital. The clinical bacterial isolates were identified to species level based by using standard microbiological methods include conventional biochemical tests [10,11] and API20E system (Biomeraux, France) at microbiological laboratories/college of Medicine/ Babylon University.

In vitro Antibacterial Effect of Cranberry Powder

Two methods were used *in vitro* to detection of antibacterial activity of cranberry powder (CP) against local bacterial isolates.

- **Agar Well Diffusion Method:** The antibacterial activity was tested using agar well diffusion method. The bacterial suspensions were diluted with normal saline. Adjust the turbidity and compared with standard McFarland tube (number 0.5) to yield a uniform suspension containing 1.5×10^{8} CFU / ml. Cotton swab was dipped and streak into adjustment suspension the entire surface of Mueller-Hinton agar (for all tested bacteria) and the plates were left for 15 minutes at room temperature to dry.

  The wells (5mm diameter) were done into inoculated media by cork borer and add 20µl of 0.5 mg/ml cranberry powder solution (the plates were performed in triplicates) in each well. All inoculated plates of the tested

organisms were then allowed to incubate at 37°C for overnight. After incubation period, the effect of CP was noted for all bacterial isolates by formation of inhibition zone[10,12].

- **Agar Dilution Method:** The two-fold serial dilutions of cranberry powder (CP) were prepared. The MIC values of CP were determined for tested organisms using a concentration ranges of dilutions from 5 to 0.0024 mg/ml was applied according to procedure described in reference[13].

The dilutions were added to molten agar medium; Muller-Hinton Agar (MHA); at temperature 45-50C and mixed well. The mixture was poured into sterile plates, and then allows solidifying at room temperature. A standardized inoculum was prepared by growing bacteria to turbidity of 0.5 McFarland tube. 1-2 µl aliquot of each inoculum was applied to the agar surface. A control plate without antibacterial agent (CP-free medium) was done. The inoculated plates were incubated at 37C for 18 hr.

In vitro Susceptibility Testing of Antibiotics

The antibacterial activity of some antibiotics was determined by using agar disc diffusion test(DDT). Agar plates were inoculated with 0.1 ml broth culture of tested organisms. The antibiotics disks (Hi-Comb, INDIA) of Ciprofloxacin(5µg), Cefotaxime(30µg), Ampicillin(10µg), Gentamicin (10µg) and Nitrofurantoin(30µg) were added in the center of agar plate (the plates were performed in triplicates). All plates of the tested organisms were then allowed to incubate at 37°C. After 18 hr of incubation, the MIC values for each antibiotic disc were determined by using Hi-comb strip (Hi-media, India) and compared with break-points which recommended by CLSI [14].

Inhibitory Effect of CP on Bacterial Adherence

Modified method [15] was used for detection of ability of bacteria for adherence to epithelial cells. The epithelial cells of bladder cavity were collected and transferred directly into sterile tubes contain PBS (PH 7) after that wash the epithelial cells by PBS, and centrifugation (at 5000 rpm for 10 minute) for three times. The filtrated epithelial cells treated with standard bacterial suspension and then with cranberry powder (CP: 1.2500 mg/ml) for different incubation times, 1-10 hr. at 37C. The mixture was washed by PBS to remove unadherent bacteria. The epithelial cells were fixed by ethanol for 15 minutes, and stained with Giemsa stain (30%) for 20 minutes. The cells were then examined under a light microscope, and the mean of tissue cells which bound more than 10 bacteria per cell was calculated.

Inhibitory Effect of CP on Biofilm Formation

In the present study, the isolates of both tested bacteria were screened for their ability to form biofilm by tissue culture plate(TCP) method as described in references [16,17] with a modification in duration of incubation which was extended to 24 hours according to producer that described by Nakao *et al*.[18].

Bacterial isolates from fresh agar plates were inoculated in Tryptic Soy broth (TSB) and incubated for 18 hrs. at 37C, and diluted 1 in 100 with fresh medium. Individual wells of sterile polystyrene 96 well-flat bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 18, 24 and 48 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (pH 7.2). Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) for 20 min. Excess stain was rinsed off by thorough washing with deionized water

and plates were kept for drying. Adherent bacterial cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria were determined with a micro-ELISA autoreader at wavelength of 595 nm($OD_{595\ nm}$). The experiment was also performed in triplicate and repeated three times when treated with 0.2 ml of 0.5 mg /ml cranberry powder solution. The data were then averaged, and the results were interpreted according to Mathur and co-workers method [17] as in Table-1.

Table 1: **Classification of Bacterial Biofilm Formation by Mathur *et al* (2006) on TCP**

Mean of OD Values	Biofilm Formation
<0.120	Non
0.120-0.240	Moderate
>0.240	High

Inhibitory Effect of CP on Bacterial Motility

The method described in reference [1] was used for detection of cranberry powder (CP) effect on bacterial motility: CP was prepared and added separately in concentration ranging (0.0-30 mg/ml) into bacterial culture media; nutrient agar and soft agar plates. All media were cultured with bacterial isolates, and then incubated at 30 C for 18 hr. After that, the effect of CP on motility activity of *Pr.mirabilis* was determined by measuring swarming diameter of and compared with swarming of positive control, whereas *E.coli* motility was determined by inhibition of growth dissemination out line of stabbing. The hanging drop method for *E.coli* isolates also done.

RESULT AND DISCUSSIONS

Antibacterial Effects of CP

Urinary tract infection can be cured by the antibiotics but it reoccurrence after some time and cause problem to the patient. The reoccurrence of UTI is a major problem especially in women. For some how the problem will be prevented by using cranberry along with the antibiotics[8].

Laboratory results of our research show that a possible effect on *E.coli* and *Pr.mirabilis* may occur from a component of the CP inhibiting cell growth. The findings of present study suggest that cranberry may have antibacterial properties.

The MIC value of CP was 1.25 mg /ml for *E.coli*, and MIC value of CP for *Pr.mirabils* was 0.1563 mg /ml shown in Table-2. The lower MIC value of cranberry powder against *Pr.mirabilis* in comparison to *E.coli* organism suggests that *Pr.mirabilis* showed greater sensitivity towards CP. Also in present study, *E.coli* was complete resistant 100% while *Pr.mirabilis* was resistant 80% to antibiotic agents that involvement in our research, except, it was sensitive 20% to Cefotaxime. For those with recurrent infections, the prolong uses of antibiotics may cause severe side effects and resistance in bacteria against antibiotics[8]. As antibiotic resistance becomes an increasing concern, alternative strategies such as cranberry consumption become more important for preventing UTIs[19].

Study in Iran by Rahbar and Diba [13] antibacterial activity of various concentrations of cranberry extract against different strains of urine isolates are showing the MIC value of cranberry extract was the same for *Enterobacter aerogenes* and *Staphylococcus aureus* (0.0391 mg/ml) whereas the MIC values were 1.2500 and 0.0195 mg/ml for *E. coli* and *E. aerogenes* respectively.

Table 2: Antibacterial Activity and MIC Values of CP Against *E. coli* and *Pr. mirabilis*

Conc. of CP (mg /ml)	*E. coli*	*Pr. mirabilis*
5.0000	-	-
2.5000	-	-
1.2500	-	-
0.6250	+	-
0.3125	+	-
0.1563	+	-
0.0781	+	+
0.0391	+	+
0.0195	+	+
0.0098	+	+
0.0049	+	+
0.0024	+	+

Potential Anti-Adhesion Properties of CP

Adherence of uropathogens to uroepithelial cells is the initial step in pathogenesis of UTI [5] so our study has concentrated on understanding how cranberries affects on adherence of *E. coli* and *Pr.mirabilis* to epithelial cells, and focusing on their beneficial effects in preventing urinary tract infections (UTIs). The ability of both genera adherence to epithelial cells was decreased when exposed to CP during *in vitro* experiment. The mean adherence of *E. coli* and *Pr.mirabilis* to epithelial cells is decrease from 20 to 1.9 bacteria per cell (Table-3).

Table 3: Estimation of CP Efficacy on Bacterial Adherence to Epithelial Cells

Conc. of CP (mg/ml)	Time of I.P (hr.)	Mean of Adherence
1:2500	1.0	20.0
	2.0	20.0
	3.0	20.0
	4.0	20.0
	5.0	3.0
	6.0	1.9
	7.0	1.9
	8.0	1.9
	9.0	1.9
	10.0	1.9

Sharma and Tiwari[8] declaring the action of cranberry in the urinary system is due to its production of hippuric acid in the urine. This causes the pH of the urine to become acidic and prevents bacteria from adhering to the bladder tissue and decrease risk of infections such as UTI or cystitis.

The article, Raz and Co-workers[5]showing that cranberries are rich in benzoic acid, which is then excreted in urine as hippuric acid. Therein followed a long period during which the usefulness of cranberry juice was thought to be based on the urinary excretion of hippuric acid, which is a bacteriostatic agent and has the potential to acidify urine. The fimbriated bacteria produce two types of adhesions (mannose sensitive and mannose resistant) that attach to receptors on uroepithelial cells [19]. Zafriri *et al.* [15] who identified two compounds in cranberries that inhibit *E.coli* and *Pr.mirabilis* adhesins. One is fructose, which inhibits the mannose-sensitive fimbrial adhesins; the other is a high-molecular-weight compound that inhibits the mannose-resistant adhesins of uropathogenic *E. coli***.**

The two compounds with antiadherence properties that prevent fimbriated bacteria from adhering to uroepithelial cells in the urinary tract[5]. Cranberry fruit contain proanthrocynadins and fructose which have similar structure as of *E.coli* so; they enter in the receptors of the uroepithelial cells and block the receptors of uroepithelial cell. Hence the *E.coli* and *Pr.mirabilis* will not able to enter in the receptors of uroepithelial cells and they are killed by the antibiotics and flush out when patient consume the glass of water[8].

Some laboratory studies, For example, Camesano *et al*.[6] suggested that growth in media containing cranberry caused *E. coli* to be unable to express their P fimbriae genes.

Inhibitory Effect of CP on Biofilm Formation

Result of present study about effect of CP on formation of bacterial biofilm showing below in Table-4. The biofilm formation of *E.coli* and *Pr.mirabilis* was much reduced.

Table 4: Estimation of CP Effect on Biofilm Formation by ELISA for Both Genera

Conc. of CP (mg/ml)	Time of I.P (hr.)	Mean of O.D by ELISA at 595 nm	Biofilm Formation
0.0 1.0	18	0.133	Moderate
5.0 10.0	24	0.99	Non
20.0 30.0	48	0.346	High

I.P: Incubation period, **O.D :** Optical density

Our study examined the effects of CP on biofilm formation of uropathogenic bacteria. The results showed that biofilm formation was reduced within 24 hours after CP exposure, and it started to increase after 48 hours. The results of study suggest that CP can be an effective preventive measure for UTIs as it inhibits adhesion and biofilm formation of uropathogenic bacteria[20]**.** Other study also has shown that cranberry extract reduce biofilms formation on uroepithelial cells[21]. The results of Tao study[2] indicate that drinking cranberry juice(CJ) can be an effective preventive measure for biofilm formation in healthy women. The anti-biofilm activity peaks between 24 and 48 hours after drinking CJ.

Inhibitory Effect of CP on Bacterial Motility

Effect of cranberry powder (CP) on motility of *E.coli* and *Pr. Mirabilis* was investigated *in vitro*. The media plates supplemented with CP at 0, 1, 5, and 10, 20, 30 mg/mL, respectively, as shown in table-3. The swarming motility of *Pr. mirabilis* and motility of *E.coli* were inhibited following CP exposure. The results show that exposure to 10 mg/ml CP completely blocked motility, even after 18 hr of incubation. CP at 5 mg/ml caused only a temporal decrease in the motility (Table-5).

Table 5: Effect of CP on Motility of *E. coli* and *Pr. mirabilis*

Conc. of CP (mg/ml)	Temp. (Ċ)	Time (hr.)	Motility of *E. coli*	Motility of *Pr. mirabilis*
0.0	30	18	+	+
1.0			+	+
5.0			+/-	+/-
10.0			_	_
20.0			_	_
30.0			_	_

+ motile, - non-motile, +/- decreased motile

Howell, 2007; the effect of cranberry on bacterial motility is interpreted by it contains tannic acids, which have been shown to inhibit the motility of several uropathogenes [20]. The tannins have also ability to inhibit binding of pili of uropathogenic bacteria such as UPEC to epithelial cells of bladder[21], while other studies[4, 22] have begun to explore the effects of cranberry materials on pathogen gene expression and phenotype as well as infectivity. This work reports the effects of cranberry powder (CP) on the motility of *Pr. mirabilis* and its expression of flaA, flhD, and ureD. The transcription of flagellin gene flaA and of flhD, the first gene of the flagellar master operon flhDC, decreased during exposure of *Pr. mirabilis* to various concentrations of CP.

CONCLUSIONS

On the basis of the experimental results and discussion, it can be postulated that the cranberry powder possesses the potent antibacterial properties.With growing antibiotic resistance; the CP is needed as alternative therapy for control on UTIs.

RECOMMENDATIONS

The flavonoids compounds of cranberry such as proanthocyanidins, flavonols and quercetin have shown possible activity as anti-cancer agents *in vitro*; however, the evidence is not definitive, and more research is needed.

ACKNOWLEDGEMENTS

I am extremely thankful to Dr. Nada K. Hindi (academic teacher in Nursing College, Babylon University), for helping and providing the needed facilities, which are essential for successful completion of the present work. I am also deeply indebted to staff and patients of Hilla hospitals for their kind assistance.

List of Abbreviations

CFU Colony forming Unit

CLSI Clinical Laboratory standard Institute

CP Cranberry powder

MIC Minimum Inhibitory Concentration

PBS Phosphate buffer solution

UPEC Uropathogenic *E.coli*

UTI Urinary Tract infection

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